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Analysis of Transmission of Novel Polymorphisms in the Somatostatin Receptor 5 (SSTR5) Gene in Patients With Autism

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Running title: Analysis of SSTR5 gene polymorphisms in autism

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Infantile autism is a pervasive developmental disorder with a strong genetic component. The mode of inheritance appears to be complex and no specific susceptibility genes have yet been identified. Chromosome 16p13.3 may contain a susceptibility gene based on findings from genome scans and reports of chromosome abnormalities in individuals with autism. The somatostatin receptor 5 (SSTR5) gene is located on chromosome 16p13.3 and is thus a positional candidate gene for autism. SSTR5 may also be a functional candidate gene for autism because somatostatin inhibits growth hormone secretion, and increased growth hormone response has been reported in some individuals with autism. Moreover, the somatostatinergic system interacts with the dopaminergic system, which has been hypothesized to be involved in the etiology of autism; in particular, somatostatin secretion is regulated by dopamine, and the dopamine D2 receptor and the SSTR5 receptor interact to form a receptor complex with enhanced functional activity. In the present study we tested whether the alleles of twelve new single nucleotide polymorphisms (SNPs) in the SSTR5 gene were preferentially transmitted using the transmission disequilibrium test (TDT) in a sample of 79 trios with autism (18 from Denmark and 61 from France). Furthermore, we combined four missense SNPs into haplotypes and searched for preferential transmission using the program TRANSMIT. No significant preferential transmission of the alleles and haplotypes of the twelve SNPs was found. Our results do not suggest the SSTR5 gene as a susceptibility gene for autism.

KEY WORDS: autism; polymorphisms; SSTR5; association; TDT

INTRODUCTION

Infantile autism is a pervasive developmental disorder characterized by abnormalities in social, communicative, and behavioral functioning and onset of symptoms before the age of three years. Although the etiology of autism is unknown, a strong genetic component has been found based on twin (Bailey et al., 1995) and family studies (Bolton et al., 1994). There is no simple mode of inheritance, and a polygenic model has been suggested with multiple interacting loci, ranging from three to more than 15 genes (Pickles et al., 1995; Risch et al., 1999). No disease genes of autism have yet been identified but susceptibility loci have been proposed on 2q31-32, 7q31-35, and 15q11-13, based on hypotheses about the pathophysiology of autism, the findings of genome scans and chromosome abnormalities among autistic individuals (for reviews, see Lauritsen and Ewald, 2001; Folstein and Rosen-Sheidley, 2001).

Several lines of research point to chromosome 16p13 as a candidate region for autism. In affected sib pair studies linkage for this region was found in three genome scans. The International Molecular Genetic Study of Autism Consortium (IMGSAC) found suggestive evidence of linkage for marker D16S3102 (23.1 cM), with a multipoint maximum lod score (MLS) of 2.93 (IMGSAC, 2001). The Paris Autism Research International Sibpair (PARIS) study also found increased allele sharing around

marker D16S3075 (23.2 cM), with a multipoint MLS of 0.74 (Philippe et al., 1999). In addition, Liu et al. (2001) reported a multipoint MLS of 1.91 at D16S2619 (28.3cM). Association between autism and tuberous sclerosis has been established (Smalley, 1998) and one of the two disease loci for tuberous sclerosis has been localized to 16p13.3 just proximal to the telomere (Povey et al., 1994), suggesting that the two disorders may share disease genes or that genes for the two disorders are localized very close to each other. Some autistic cases with chromosome abnormalities in the same region have been reported as well. Hebebrand et al. (1994) reported a case with a partial trisomy 16p (dup(16)p13.1-pter) diagnosed with both autistic disorder and Tourette syndrome. Philippe et al. (1999) excluded a sib-pair from the genome-wide scan because one of the probands had a microduplication of 16p13. This region also contains the somatostatin receptor 5 (SSTR5) gene (Takeda et al., 1995; Deloukas et al., 1998), which is therefore a positional candidate gene for autism.

SSTR5 is expressed in the brain, especially in the pituitary gland, and in the fetal hypothalamus (Patel et al., 1995). The SSTR5 receptor has a high affinity for somatostatin, a multifunctional neuropeptide widely distributed throughout the central nervous system that acts in the anterior pituitary gland to inhibit the secretion of growth hormone. Recent reports of increased growth hormone response to the 5-HT1d receptor agonist sumatriptan in some individuals with autism (Hollander et al., 2000; Novotny et al., 2000) may be due to dysfunction of the somatostatergic system, making SSTR5 interesting as a functional candidate gene for autism. Furthermore, the somatostatergic system interacts with the dopaminergic system, which has been hypothesized to be involved in autism. In particular, dopamine seems to exert both stimulatory and inhibitory effects on the secretion of somatostatin (Gillies, 1997). Moreover, it has recently been shown that the SSTR5 receptor interacts physically with the long form of the dopamine D2 receptor through hetero-oligomerization to create a novel receptor with enhanced functional activity (Rocheville et al., 2000). This receptor complex, simultaneously occupied by two ligands, appears to be the most active signaling form. The dopaminergic system has been implicated in the etiology of autism because low levels of serum dopamine β -hydroxylase have been reported in some families with autistic children (Lake et al., 1977) and because dopamine D2 receptor antagonists reduce self-injurious behavior in some autistic cases (King, 2000).

We have identified twelve new single nucleotide polymorphisms (SNPs) in the SSTR5 gene, five in the 5'-promoter region and seven in the coding region (Nyegaard et al., 2002). In this study, we examined possible linkage disequilibrium between autism and the SSTR5 gene using the transmission disequilibrium test (TDT) (Spielman and Ewens, 1998). We also constructed haplotypes of functional SNPs and tested for excess transmission using the TRANSMIT program (Clayton, 1999).

MATERIALS AND METHODS

Family Recruitment and Diagnostic Assessments

For this study Danish and French probands and their parents were ascertained and diagnosed as described elsewhere (Lauritsen et al., 2002). Briefly, Danish probands were recruited at child

psychiatric hospitals and assessed with the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994) and the Autism Diagnostic Observation Schedule (ADOS) or the more recent ADOS-Generic (ADOS-G) (Lord et al., 2000). French families were recruited at an outpatient university clinic for children with autism in Paris (Hôpital Robert Debré). The diagnosis was confirmed using the ADI-R. Subjects with known etiologies of autism were excluded. The study was approved by the ethical committees in Denmark and France. Informed consent forms were completed by the parents.

A total of 79 families with one child with infantile autism and both parents, were included. Of these families, 18 were Danish, with a male-female ratio of 3.5:1, and 61 were French, with a male-female ratio of 2.4:1. All patients were of Caucasian descent. Mean age of the Danish probands was 11 years (age range, 3-30), and mean age of the French probands was 12 years (age range, 4-27).

Laboratory Procedures

Blood samples were collected from children and their parents and DNA was extracted from lymphocytes by standard procedures. Genotyping was performed essentially as described previously (Nyegaard et al., 2002). Briefly, two overlapping fragments (A and B), together containing the total set of SNPs, were amplified by PCR. PCR primer sequences were Afor:5'-TCTCTGGACCTTGTGCCAGC, Arev:5'-GGCCACTGCCAGGTTGAGAA, Bfor:5'-ATGTGCTGGTTCAGGGACTC, and Brev: 5'-TGACTGGCAGGTCATGGGTG. After PCR, fragments A and B were pooled and purified from primers and unincorporated dNTPs by adding SAP (USB, Ohio, USA) and ExoI (New England Biolabs, Massachusetts, USA). Single basepair extension (SBE) was carried out using SNaPshot mix (Applied Biosystems, Foster City, USA) and a mixture of SNP primers, each designed to terminate on the base 5' to an SNP and to avoid containing any neighboring SNPs. The length of the primers was adjusted to 18, 22, 24, 26, 30, 32, 34, 36, 38, 40, 42, and 46 basepairs by adding a random sequence to the 5'-end. After the SBE reaction, excess ddNTPs was removed by adding SAP. Samples were assayed by electrophoresis on an ABI 377 sequencer using a 12% denaturing polyacrylamide gel.

The 12 SNPs were identified within a 3.3 Kb genomic segment by direct sequencing of five individuals with bipolar disorder and two healthy individuals (Nyegaard et al., 2002). Five of the SNPs (SNP1, SNP3, SNP13, SNP4, SNP5) were identified in the 2.2 kb region immediately 5' upstream of the SSTR5 gene, and the other seven were identified in the 1092 coding region of the SSTR5 gene, which is intronless. The positions of SNP1, SNP3, SNP13, SNP4, and SNP5 are 71, 124, 593, 963, 1457, and 1797, respectively, in Genbank accession no. AF152962 (GI:5031446). The positions of SNP6, SNP7, SNP8, SNP9, SNP10, SNP11, SNP12 are 192, 205, 375, 623, 683, 1054, and 1094, respectively, in Genbank accession no. D16827 (GI:487683). In the NCBI SNP database (<http://www.ncbi.nlm.nih.gov.SNP/>), rs169068 and rs642249 correspond to SNP11 and SNP12, respectively. Most pairs of SNPs were in strong linkage disequilibrium, including all adjacent SNPs except SNPs 3-4 and 4-5.

Statistical Analysis

Probands and parents were checked separately for Hardy-Weinberg equilibrium using a likelihood ratio chi-squared test. The transmission disequilibrium test (TDT) was used to compare transmission of each of the two alleles of twelve SNPs of the SSTR5 gene using the χ^2 test (Spielman and Ewens, 1998). In the TDT, we only included trios with known genotypes of the proband and both parents. For some of the trios the SNPs could not be genotyped so in order to extract additional information, the program TRANSMIT (<http://watson.hgen.pitt.edu/docs/transmit.html>) (Clayton, 1999) was used to estimate the frequencies of the alleles of every SNP and to check for excess transmission of multilocus haplotypes. One of the advantages of TRANSMIT is the ability to estimate missing genotypes of parents and deal with unknown phases.

Subsets of SNPs were chosen for construction of haplotypes based on knowledge about their possible function (Nyegaard et al., 2002). In the promoter region, one haplotype consisting of SNP3 and SNP5 was constructed because these two SNPs were found within the core sequences of two different promoter element binding sites and the haplotype may therefore affect transcription level of the SSTR5 gene. In the coding region, a second haplotype was constructed by combining SNP6 and SNP7, both positioned in the transmembrane region and introducing a hydrophobic to hydrophobic amino acid change (Leu48Met and Ala52Val respectively). Even though these are only minor protein chemical changes, they may still influence binding affinity or receptor stability. A third haplotype was constructed with SNP8 and SNP11, both non-conservative amino acid changes involving Proline (Pro109Ser and Pro335Leu respectively). Proline is often of structural importance and it is possible that the Pro substitutions involve a stability or structure change of the receptor. Finally, a haplotype consisting of the four SNPs coding for an amino acid substitution was constructed (SNP6, SNP7, SNP8, and SNP11). We have previously shown that these subsets of SNPs except SNP3 and SNP5 are in strong linkage disequilibrium (Nyegaard et al., 2002).

RESULTS

Among cases, the test for Hardy-Weinberg equilibrium showed no deviation for any of the SNPs tested (p-values from 0.45 to 0.88), except for SNP12 (p = 0.04) (not shown in detail). Among parents, no deviation from Hardy-Weinberg equilibrium was found (p-values from 0.17 to 0.78) (not shown). The TDT showed no significant transmission distortion of any alleles of the twelve SSTR5 gene polymorphisms (Table I). Moreover, no excess transmission of any alleles of the SSTR5 SNPs (Table II) or of the constructed haplotypes of the SNPs (Table III) was found using TRANSMIT.

DISCUSSION

To our knowledge, this is the first study to investigate the SSTR5 gene as a candidate gene for autism. The study design using subjects with infantile autism and their parents was chosen to avoid population stratification which could be a problem since our sample consisted of both French and Danish

probands. We found no evidence of association between autism and twelve SNPs of the SSTR5 gene in 79 autistic cases from Denmark and France and their parents. This may be due to low allele frequency of some of the SNPs genotyped and they need to be studied in additional samples. We cannot, however, exclude the SSTR5 gene from involvement in the etiology of autism since the gene may contain SNPs associated with autism and undetected by our research group because we only tested for mutations in seven individuals. Also, we did not test for all possible haplotypes but decided to focus on the functions of the SNPs and therefore other associated haplotypes may be missed although this is less likely considering the strong linkage disequilibrium in the gene.

Because new results have revealed that SSTR5 and DRD2 form functional heterodimers (Rocheville et al., 2000), we also checked the Danish and French cases for the following five mutations in the DRD2 gene: Val96Ala (Gejman et al., 1994), Val154Ile (Klein et al., 1999), Pro310Ser (Gejman et al., 1994), Ser311Cys (Itokawa et al., 1993), and Thr351Ala (available via the NCBI SNP database (<http://www.ncbi.nlm.nih.gov/SNP/>), SNP ID rs1110977ref). Not surprisingly, we were unable to find any of the five DRD2 missense mutations among cases since they are all very rare.

In conclusion, although the SSTR5 gene was not found to be a susceptibility gene for autism in this study, a possible association between SSTR5 and autism cannot be totally dispelled due to the rarity of some of the SNP alleles in relation to the sample size. Moreover, it cannot be ruled out that the SSTR5 gene is a susceptibility gene in a subgroup of autistic individuals.

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TABLE I. TDT of 12 SNPs in the *SSTR5* Gene and Autism

SNPs	Position	Transmitted	Not Transmitted	TDT χ^2	<i>P</i> value
SNP1	Promoter	23	18	0.61	0.43
SNP 3	Promoter	25	16	1.98	0.16
SNP 13	Promoter	21	15	1.00	0.32
SNP 4	Promoter	11	8	0.47	0.49
SNP 5	Promoter	38	32	0.51	0.47
SNP 6	Coding (Leu48Met)	9	6	0.60	0.44
SNP 7	Coding (Ala52Val)	3	9	3.00	0.08
SNP 8	Coding (Pro109Ser)	3	8	2.27	0.13
SNP 9	Coding	3	3	0	1.00
SNP 10	Coding	7	7	0	1.00
SNP 11	Coding (Pro335Leu)	40	31	1.14	0.29
SNP 12	Coding	5	4	0.11	0.74

TABLE II. Test for Excess Transmission of Alleles of 12 SNPs in the *SSTR5* Gene and Autism Using TRANSMIT

SNPs	Position	Allele 1	Allele 2	χ^2	<i>P</i> value
SNP 1	Promoter	0.81	0.19	1.02	0.31
SNP 3	Promoter	0.82	0.18	1.35	0.25
SNP 13	Promoter	0.82	0.18	1.16	0.28
SNP 4	Promoter	0.92	0.08	0.12	0.73
SNP 5	Promoter	0.40	0.60	1.49	0.22
SNP 6	Coding (Leu48Met)	0.93	0.07	1.13	0.29
SNP 7	Coding (Ala52Val)	0.96	0.04	2.17	0.14
SNP 8	Coding (Pro109Ser)	0.96	0.04	2.54	0.11
SNP 9	Coding	0.98	0.02	0.10	0.76
SNP 10	Coding	0.95	0.05	0.12	0.73
SNP 11	Coding (Pro335Leu)	0.44	0.56	2.08	0.15
SNP 12	Coding	0.97	0.03	0.01	0.94

TABLE III. Test for Excess Transmission of Multilocus Haplotypes of 12 SNPs in the *SSTR5* Gene and Autism Using TRANSMIT

	Haplotype	Estimated frequency of haplotype*	χ^2	df	P value
SNP 3-5	1 1	0.35	0.99	1	0.32
	2 1	0.05	0.41	1	0.52
	1 2	0.47	0.001	1	0.97
	2 2	0.12	3.18	1	0.07
	Global		3.78	3	0.29
SNP 6-7	1 1	89	0.03	1	0.86
	2 1	7	1.13	1	0.29
	1 2	4	2.17	1	0.14
	Global		3.09	2	0.21
SNP 8-11	1 1	0.40	0.76	1	0.38
	2 1	0.036*	2.59	1	0.11
	1 2	0.57	2.06	1	0.15
	Global		3.45	2	0.18*
SNP 6-7-8-11	1 1 1 1	0.33	2.15	3	0.14
	2 1 1 1	0.06	1.87	3	0.17
	1 2 2 1	0.039*	3.28	3	0.07
	1 1 1 2	0.56	1.94	3	0.16
	2 1 1 2	0.004*	0.9995	3	0.32
	1 2 1 2	0.003*	0.96	3	0.33
	Global		9.26	5	0.099*
Global			6.69	3	0.08

* P values of test of rare haplotypes, with frequencies less than 4%, are indicated with an asterisk.